In vitro assessment of sperm from bulls of high and low field fertility

A. Al Naib[a], J.P. Hanrahan[b], P. Lonergan[a], S. Fair[c,*]

[a]*School of Agriculture, Food Science and Veterinary Medicine, College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland.* [b]*Teagasc, Animal Production Research Centre, Athenry, Co. Galway, Ireland.* [c]*Department of Life Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland.*

*Corresponding Author:

Dr Sean Fair, Department of Life Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland. Tel: + 353 61 202548, Fax: + 353 61 331490, E-mail sean.fair@ul.ie

Abstract

The aim of this study was to investigate the reasons for differences in field fertility of bulls following insemination with frozen-thawed semen. The study was carried out in two separate parts over two years and comparisons were made between 5 high and 4 low fertility Holstein Friesian bulls as determined by their either 90 day non-return rate (Year 1) or calving rate (Year 2). Two high fertility Limousin bulls were included in Year 1 for comparative purposes. The ability of sperm from each bull to penetrate artificial mucus was assessed (Year 1 = 7 replicates; Year 2 = 5 replicates). Glass capillary tubes (2 per bull per replicate) were filled with artificial mucus and incubated with sperm stained in 1% Hoechst 33342 for 30 min at 37 ^{o}C. The number of sperm were subsequently counted at 10 mm intervals along the tube between 40 and 80 mm markers. Sperm mitochondrial activity of each bull was assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (4 replicates in each year). Sperm were incubated with MTT for 1 h at 37^{o}C following which the absorbance of formazan was read using a spectrophotometer. Sperm viability after thawing was assessed for each bull using a live/dead sperm viability kit (Year 1 = 3 replicates; Year 2 = 4 replicates). A minimum of 250 cells were assessed per bull in each replicate and classified as either live or dead. Finally, the ability of sperm to fertilise oocytes *in vitro* and their ability to develop to blastocyst stage embryos were assessed (5 replicates in each year involving 220 to 306 oocytes per bull). Data transformation to normalise residuals was required for mucus sperm penetration (square root) and IVF (cleavage and blastocyst rate) results (arcsin). The mean number of sperm counted at each 10 mm mark between 40 and 80 mm was higher in the high fertility (56.0; 95%CI 39.5 to 75.3) compared to the low fertility

(42.9; 95% CI 29.3 to 59.1) Holstein Friesian bulls but the difference did not reach formal significance (P = 0.09). Fertility status had no effect on the ability of sperm to reduce MTT to formazan (mean absorbance 0.34 ± 0.051 and 0.30 ± 0.044) or on the percentage of live sperm per straw (mean 47.3 ± 5.47 and 32.4 ± 4.66) for high and low fertility Holstein Friesian bulls respectively. Oocyte cleavage rate following insemination with sperm from high fertility Holstein Friesian bulls was significantly higher than with sperm from low fertility Holstein Friesian bulls [76.7% (95%CI 60.9 to 89.4) and 55.3 (95%CI 40.4 to 69.7) respectively, P = 0.04]. There was no significant effect of bull fertility on blastocyst rate [34.7% (95%CI 21.1 to 49.6) and 24.2 % (95%CI 14.1 to 36.0) for the high and low fertility Holstein Friesian bulls, respectively; P = 0.2]. In conclusion, sperm from high fertility bulls tended to be more effective in penetrating artificial mucus and to have an increased ability to fertilise oocytes *in vitro;* however, once fertilisation occurred subsequent embryo development was not significantly affected by fertility status.

Keywords: Bull semen, field fertility, artificial mucus, IVF, MTT

1. Introduction

The introduction of artificial insemination (AI) to the dairy industry in the 1950s revolutionised cattle breeding and has displaced natural service as the preferred method of breeding in most developed countries. More recent developments in molecular biology have enabled the genomic selection of young elite bulls for inclusion in AI programmes [1]. This technology is now commercially available and coupled with AI allows for faster genetic progress [2]. However, one of the problems

of using young bulls in artificial insemination programmes is that, by definition, their field fertility has not been proven. The fertility of a bull has traditionally been evaluated by test inseminations in the field and while this method is considered reliable, it is expensive and time-consuming [3]. Consequently, it would be of benefit to the cattle breeding industry to have an accurate, simple and efficient *in vitro* method of predicting the potential fertility of semen, where aspects such as time, cost and practicability are considered. In order to develop such a test it is first necessary to understand why frozen-thawed semen from some bulls results in a higher pregnancy rate than that from other bulls. Several hypotheses exist as to possible reasons for such differences. Frozen-thawed sperm from low fertility bulls may exhibit an abnormal change in morphology or metabolic activity, be unable to transverse the female reproductive tract to the site of fertilisation in sufficient numbers, or their ability to fertilise the oocyte or yield a developmentally competent embryo may be impaired.

Conventional *in vitro* evaluation of semen quality following the freeze thaw process, such as the assessment of concentration, motility and morphology are of limited value in assessing field fertility [4]. Various fluorescent staining techniques have also been used to evaluate sperm viability [5,6,7], capacitation status [8,9], membrane integrity [10], chromatin integrity [11], acrosome status [5,12] and mitochondrial activity [5,13], and while these measurements are useful for *in vitro* assessment of sperm they have limited ability to predict field fertility. The rate at which specific stains can be reduced by the mitochondria of sperm has also been used as an assessment of metabolic status [14]. It has been suggested that MTT (3- (4,5-Dimethylthiazol-2-yl) - 2,5- diphenyl- tetrazolium bromide) may be used as a reliable indicator for bovine [14] and equine [15] sperm fertility. MTT is a yellow, water soluble tetrazolium salt which

is reduced to water-insoluble purple formazan crystals in the mitochondria of living cells. The amount of formazan can be measured spectrophotometrically and therefore gives an estimate of the number of living cells in a sample. This method was first reported by Mosmann [16], who considered it to be a simple and inexpensive method to assess viability, and has been used widely on many cells types [14,17,18,19].

It has been reported that *in vivo* barriers to sperm transport can be mimicked *in vitro* using sperm migration tests as a tool to examine sperm quality [20,21]. These tests have been used for determination of sperm function in humans [22], goats [23] and bulls [24,25]. Recently, artificial mucus composed of hyaluronic acid [26] or polyacrylamide gel [25] has been used as a suitable and more consistent alternative to natural mucus. On the other hand other authors have suggested that there is no relationship between mucus penetration and field fertility [21,27,28].

Some authors have reported that *in vitro* fertilisation (IVF) can be used as a tool to predict the field fertility of a bull or to discriminate among bulls of different field fertility in terms of both cleavage and blastocyst formation rates [29,30,31,32]. Others have reported a correlation between field fertility and cleavage rate alone [33,34] or blastocyst formation rate alone [35,36], while some authors have reported that IVF is not a useful predictor of field fertility [37,38,39].

The aim of the present study was to investigate whether differences in field fertility of bulls is reflected in differences (i) in the metabolic activity of sperm, in terms of their ability to penetrate mucus, their mitochondrial activity as well as their ability to

survive the freeze-thaw process or (ii) in the ability to fertilise oocytes and produce viable embryos.

2. Materials and Methods

2.1 Experimental Design

All bulls used in this study were located in the National Cattle Breeding Centre (NCBC), Enfield, Co Meath, Ireland and were used for commercial AI in Ireland. Semen was diluted and then frozen in 0.25 mL straws, each containing approximately 80 x 10^6 spermatozoa per 1 mL. The study was carried out over two years and involved a separate sample of bulls each year. In Year 1, semen from six bulls was used of which three bulls (1 Holstein Friesian and 2 Limousin) were classified as having 'high fertility' and three Holstein Friesian bulls were classified as having 'low fertility'. Given the different breeds in the high fertility group, the experiment was repeated in Year 2 using semen from five Holstein Friesian bulls of which three were classified as having 'high fertility' and two were classified as having 'low fertility'. In Year 1 the field fertility of the 6 bulls was calculated as 90-day non-return rate (NRR; Table 1). Due to an updated recording system used by the NCBC NRR was not available for the bulls in Year 2. Instead the field fertility was available as pregnancy rate adjusted for various factors, including semen type (frozen, fresh), parity of cow, month of service, day of the week when serviced, individual cow effects and AI technician effect, and weighted for number of service records [44].

2.2. Assessment of sperm motility and concentration

Straws were thawed at 37 ^{o}C for 30 s in a water bath and semen was held in a 15 mL polypropylene conical tube during processing. As a quality control check a 5 µL

sample was placed on a pre-warmed slide and motility was subjectively assessed on a scale of 0 to 5 (0 = no motility, 5 = progressively motile) for each bull prior to each replicate in each experiment. Sperm concentration was assessed for each bull using a haemocytometer and dilutions were performed using Tyrode's albumin lactate pyruvate (TALP) media as appropriate [40].

2.3. Mucus penetration test

The ability of sperm to penetrate artificial mucus was evaluated using the method described by O'Hara et al. [41]. Artificial mucus was prepared by diluting a solution containing sodium hyaluronate (MAP-5, Labstock MicroServices, Ireland) with phosphate-buffered saline to give a final concentration of 6 mg sodium hyalorunate per 1 mL.

The penetration test was replicated (7 times in Year 1 and 5 times in Year 2). For each replicate one straw per bull was thawed and held in a water bath at 37 ºC for processing. Each bull used in a given year was represented in each replicate in that year and was kept separate throughout. Semen was diluted to a final sperm concentration of 10×10^6 per mL in TALP medium containing Hoechst 33342 fluorescent stain (10 mg/mL in 2.3% sodium citrate) and incubated at 37 ºC for 5 min to ensure uptake of the stain.

Flattened capillary tubes (0.3 mm x 3.0 mm x 100 mm; Composite Metal Services Ltd, UK) were marked at 10 mm intervals between 10 and 80 mm. These were then filled with artificial mucus using an adapted 5 mL syringe. Two capillary tubes were placed vertically in a 1.5 mL eppendorf containing a 100 µL aliquot of the stained

sperm from one bull. Therefore, in each replicate each of the bulls were represented by two capillary tubes. The tubes were incubated in a dry oven for 30 min at 37 ºC. After incubation the tubes were placed on a hotplate at 45 ºC for 1 min to immobilize the sperm. Sperm were counted from wall to wall across the tube, within the width of a single field of view, at each 10 mm interval using a fluorescent microscope (40x; Olympus BX 60). Because the test yielded an excessive number of sperm at 10, 20 and 30 mm positions, which made an accurate count impossible, these data points were excluded from the analysis.

2.4. Assessment of sperm cell viability

Sperm viability status of each bull was assessed using a live/dead sperm viability kit (SYBR 14/ Propidium Iodide (PI); Molecular Probes L-7011). One straw was analysed per bull in each of three replicates in Year 1 and in each of four replicates in Year 2. Semen was diluted 1:30 with Synthetic Oviduct Fluid (SOF) medium and 5 µL of SYBR-14 stain was added to 995 µL of the diluted sample in an eppendorf to give a final SYBR-14 concentration of 100 nM. The stained sample was vortexed and incubated for 10 min at 37 °C in 95% O_2 and 5% CO_2. Following this, 5 µL of PI was added to give a final concentration of 12 µm PI and the sample was then incubated for an additional 5 min at 37 °C as above. Two aliquots, each of 4 µL were then placed on a pre-warmed slide, covered with a cover slip and viewed using a fluorescent microscope (40x; Olympus BX 60). A minimum of 250 cells were assessed per droplet and classified as either live (stained green with SYBR 14) or dead (stained red with propidium iodide).

2.5. MTT Assay

The MTT assay was performed according to the method of Aziz [14]. Four replicates were performed in each of Years 1 and 2. One straw per bull was thawed, motility was assessed and sperm were diluted to 20 x 10^6 per mL in PBS + 3% bovine serum albumin (BSA) and 100 µL of this suspension was aliquoted into a well of a 96-well plate (2 to 3 wells per bull per replicate). MTT (10 µL of a 5 mg/mL solution) was added to each well and the plate was incubated at 37 ^{o}C for 1 h in a dry oven. Following the incubation dimethyl sulfoxide (80 µL per well) was added; the plate was covered in tinfoil and agitated for 1 min to dissolve any formazan crystals. The optical density of the samples was then read using a spectrophotometer at both 550 (maximum absorbance of formazan) and 690 nm (to account for background absorbance) and the results subtracted from each other.

2.6. In vitro fertilisation

2.6.1. Oocyte collection and in vitro maturation (IVM)

Cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of slaughtered heifers. A total of 5 batches of COCs were used each year and all bulls within a year were evaluated in each batch (replicate) for that year. After four washes in PBS supplemented with pyruvate (36 µg/mL), gentamycin (50 µg/mL) and BSA (0.5 mg/mL; Sigma, St Louis, MO), groups of up to 50 COCs were placed in 500 µL maturation medium in four-well dishes (Nunc, Roskilde) and cultured for 24 h at 39 ^{o}C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF) [42].

2.6.2. Sperm preparation and in vitro fertilisation

For IVF, COCs were washed four times in fertilisation medium before being randomly assigned to treatment and transferred, in groups of up to 50, into four-well dishes containing 250 µL fertilisation medium (Tyrode's medium with 25 mmol bicarbonate, 22 mmol sodium lactate, 1 mmol sodium pyruvate and 6 mg/mL fatty acid-free BSA). In addition, heparin–sodium salt (10 µg/mL; Calbiochem, San Diego, CA) was added. Motile spermatozoa were obtained by centrifugation of frozen–thawed semen. One straw per bull in each replicate was centrifuged on a discontinuous Percoll (Pharmacia, Uppsala) density gradient (2.5 mL 45% (v/v) Percoll over 2.5 mL 90% (v/v) Percoll) at 2000 g for 9 min at room temperature. Viable spermatozoa collected at the bottom of the 90% fraction were washed in Hepes-buffered Tyrode's medium and pelleted by centrifugation at 1000 g for 5 min. The spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of fertilisation medium to give a concentration of 2×10^6 spermatozoa per 1 mL. A 250 µL aliquot of this suspension was added to each fertilisation well to obtain a final concentration of 1×10^6 spermatozoa per 1 mL. The plates were incubated for 20 h at 39 ^{o}C under an atmosphere of 5% CO_2 in air with maximum humidity.

2.6.3. Embryo culture after fertilisation

At approximately 20 h after insemination, presumptive zygotes were denuded by gentle vortexing and washed four times in PBS before they were transferred to 25 µl culture droplets of SOF (25 embryos per droplet) [43] under mineral oil. FCS (10%) was added 24 h after placement in culture. The dishes were incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39 ^{o}C. Cleavage rates were recorded at 48 h post insemination and the proportion of embryos developing to the blastocyst stage was recorded from Day 6 to 8.

241

242 *2.7. Statistical analysis*

243 Data from Years 1 and 2 were pooled for statistical analysis using mixed model
244 procedures [45]. While data for the two Limousin bulls evaluated in Year 1 were
245 retained in the analyses, the effect of fertility status was evaluated from the contrast
246 between 'high' and 'low' status Holstein Friesian bulls.

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248 The number of sperm counted per capillary tube at each 10 mm point between 40 and
249 80 mm, inclusive, was averaged and this value, after square root transformation to
250 normalise the residuals, was analysed using a model with year, and breed by fertility
251 class as fixed effects and bull and year x replicate as random terms. Thus, the error
252 term for evaluating the difference between fertility classes had 7 degrees of freedom.
253 Data on the percentage of live sperm and MTT were analysed using the same model
254 but without any transformation. The data from IVF [cleavage rate and blastocyst rate
255 (number of blastocysts relative to the number of cleaved oocytes)] were subjected to
256 arcsin transformation prior to analysis using the same model as for the other variables.
257 Where transformations were involved the means are presented after back-
258 transformation along with associated 95% confidence intervals (95%CI). In the other
259 cases least squares means are presented with associated standard errors.

260

261 **3. Results**

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263 *3.1. Mucus penetration test*

264 Sperm from high fertility Holstein Friesian bulls penetrated artificial mucus in greater
265 numbers than sperm from low fertility Holstein Friesian bulls, however, this

difference did not reach significance (P = 0.09). The mean number of sperm at each 10 mm point was 56.0 (95%CI 39.5 to 75.3) and 42.9 (95%CI 29.3 to 59.1) for high and low fertility Holstein Friesian bulls, respectively (Fig. 1). The corresponding value for the high fertility Limousin bulls was 90.8 (95%CI 52.8 to 111.5).

3.2. Assessment of sperm cell viability and mitochondrial metabolic activity

The percentage of live sperm for the high and low fertility Holstein Friesian bulls was 47.3 ± 5.47 and 32.4 ± 4.66 respectively; the difference did not reach statistical significance (P = 0.08). The percentage of live sperm for the high fertility Limousin bulls was 29.1 ± 8.22. In addition, there was no difference in the ability of sperm from high and low fertility Holstein Friesian bulls to reduce MTT to formazan (mean absorbance 0.34 ± 0.051 and 0.30 ± 0.044 for the high and low fertility Holstein Friesian bulls respectively; P = 0.6). The mean absorbance for the high fertility Limousin bulls was 0.30 ± 0.072.

3.3. In vitro fertilisation

A higher percentage of oocytes were cleaved following insemination with sperm from high fertility compared with sperm from low fertility Holstein Friesian bulls: 76.7 (95%CI 60.9 to 89.4) and 55.3 (95%CI 40.4 to 69.7) respectively, (P = 0.04; Fig. 2). There was no evidence for an effect of fertility on the percentage of cleaved oocytes that developed to the blastocyst stage: 34.7 (95%CI 21.1 to 49.6) and 24.2 (95%CI 14.1 to 36.0) for the high and low fertility Holstein Friesian bulls, respectively (P = 0.2; Fig. 2). The cleavage and blastocyst rates for the high fertility Limousin bulls were 63.7 (95%CI 39.1 to 46.4) and 25.1 (95%CI 8.8 to 46.4), respectively.

4. Discussion

Understanding the basis for differences in field fertility between individual bulls is an important objective towards developing a predictive *in vitro* test of male field fertility. In the current study, spermatozoa from bulls with superior field fertility displayed an increased ability to fertilise oocytes *in vitro* as well as an increased ability to penetrate artificial mucus. However, these differences were not associated with differences in sperm mitochondrial metabolic activity, as determined by the MTT assay.

Sperm, *in vivo*, must penetrate viscous mucus secreted by the mucosal epithelial layer as they navigate towards the site of fertilisation in the ampulla of the oviducts. This represents a major barrier and can be mimicked by *in vitro* migration tests to evaluate sperm quality [20,21]. In the current study, sperm from high fertility bulls tended to be more capable of penetrating artificial mucus than those from low fertility bulls, however, this did not reach formal significance. The reason why sperm from high fertility bulls penetrated artificial mucus in greater numbers was not due to the increased mitochondrial metabolic activity, as determined by the MTT assay. In addition, it was not due to a greater number of live sperm in the incubated sample as was shown by the percentage live following staining. The number of live sperm in all samples, from both high and low fertility bulls, was low reflecting the relatively poor survivability of bull sperm following the freeze-thaw process.

The ability of sperm to reach the site of fertilisation represents only part of their challenge on the journey to establishing a viable pregnancy. They must then penetrate and fertilise the oocyte and produce a viable embryo. The outcome of IVF has

316 previously been positively correlated with field fertility in cattle [29,30,32,46-48] and
317 sheep [49]; however, others found no such relationship [37-39]. In the current study,
318 the significant differences in cleavage rate but not blastocyst rate, following selection
319 of motile sperm, indicates that the freeze-thaw process inherently reduces the
320 fertilising ability of motile sperm of low fertility bulls to a greater degree but once
321 fertilisation has been achieved there is no evident difference between bulls in the
322 ability of their sperm to yield developmentally competent embryos.
323
324 In conclusion, sperm from low fertility bulls exhibited a reduced ability to penetrate
325 mucus as well as to fertilise oocytes *in vitro.* However, once fertilisation occurred
326 subsequent embryo development to the blastocyst stage proceeded normally
327 irrespective of bull fertility level. These differences were not due to any difference in
328 the mitochondrial metabolic activity of the sperm, as determined by the MTT assay,
329 but may be due to other sub-cellular damage caused by the freeze thaw process.
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338 **References**

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Table 1: *In vivo* fertility data of bulls of high and low fertility as determined by non return rate (NRR; Year 1) and pregnancy rate (Year 2).

Year	Bull number	Breed of bull	Number of inseminations	90 day NRR (%)	Pregnancy rate (%)	Fertility status
1	2	Holstein	688	75.3		High
1	5	Holstein	1490	65.4		Low
1	6	Holstein	1308	65.1		Low
1	4	Holstein	3594	66.2		Low
1	1	Limousin	483	75.8		High
1	3	Limousin	720	75.6		High
2	7	Holstein	637		51.8	High
2	8	Holstein	3055		54.7	High
2	9	Holstein	430		53.3	High
2	10	Holstein	364		26.6	Low
2	11	Holstein	601		23.1	Low

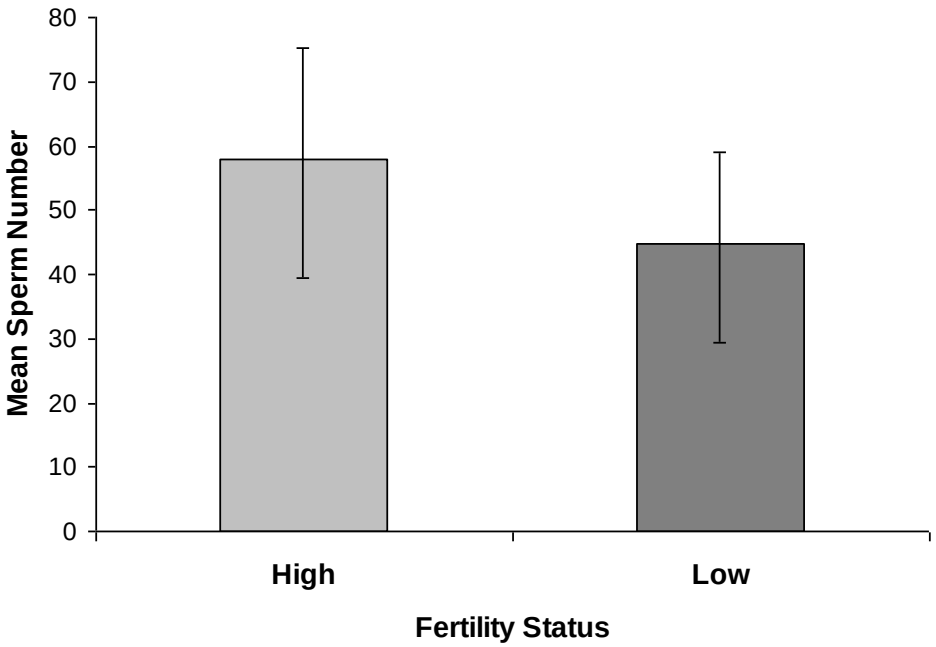

Fig.1. The mean (back transformed) number of sperm in artificial mucus (counted per 10 mm mark between 40 and 80 mm) for high and low fertility Holstein Friesian bulls. Vertical bars represent the 95% confidence interval.

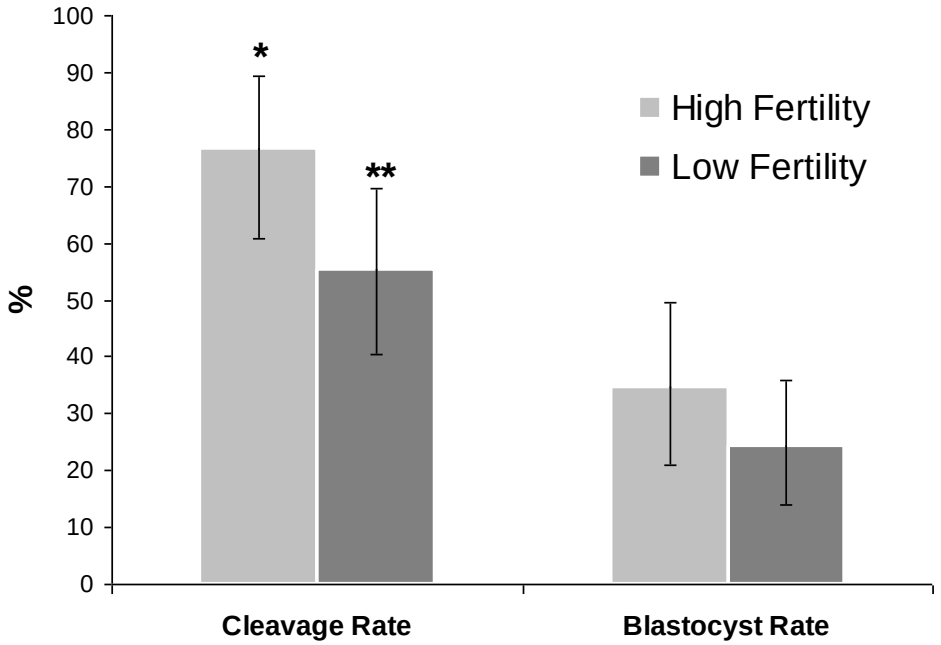

Fig.2. Cleavage and blastocyst rates following *in vitro* fertilisation of oocytes with sperm from high and low fertility Holstein Friesian bulls. Blastocyst rate was assessed on Day 8 and is expressed as a % of cleaved oocytes. Vertical bars represent the 95% confidence intervals. *Bars with different superscripts differ significantly.